Multi-Tube Fermentation (MTF) Procedure

Procedures outlined are taken from Standard Methods for the Examination of Water and Wastewater, 18th Edition (1992). Other reference material that may be of use includes:

- Microbiological Methods for Monitoring the Environment, Water and Wastes (EPA-600/8-78-017) (1978)
- Difco Manual, Tenth Edition (1984)
- Distribution System Bacteriological Sampling Control and Guidelines, AWWA (1978)

These and other applicable materials are kept on file in the laboratory.

Media Preparation

Careful media preparation is necessary for meaningful bacteriological testing. Attention must be given to the quality, measuring, mixing and sterilization of the ingredients. The purpose of this care is to assure that if the bacteria being tested for are indeed present in a sample, every opportunity is present for their development and ultimate identification. Much bacteriological identification is done by noting changes in the medium; consequently, the composition of the media must be standardized. Much of the tedium of media preparation can be avoided by purchase of dehydrated media (Difco, BBL, or equivalent). The operator is advised to make use of these products or, if only a limited amount of testing is to be done, consider using tubed, prepared media.

Quality Control

All glassware must be thoroughly cleaned using a suitable detergent and hot water (70°C), rinsed with hot water (80°C) to remove all traces of residual detergent, and finally rinsed with distilled water **12 TIMES**. This is accomplished during the run cycle of the dishwasher.

Use only distilled water, which has been tested and found free from traces of dissolved metals for preparation of culture media.

For each batch of media that is made, the following checks will be run: a sterile, a positive (+), and a negative (-). These will be inoculated as soon as possible after the sterilized media has cooled. The pH also will be checked after autoclaving and recorded in the media logbook.

Stock Cultures

In order to run the above quality check, five different stock cultures are kept on hand.

Enterobacter aerogenes
Escherichia coli
Pseudomonas aeruginosa

To start a new set of cultures, prepare 100ml of Tryptic Soy Broth (3.00g of dehydrated media in 100ml of water). Fill each of five disposable culture tubes (18x150mm) with 15ml of prepared media and pipette remaining 25ml of TSB into a 100ml beaker. Cover containers and autoclave at 121°C for 15 minutes (45-min. cycle). When cooled, record pH of media in beaker.

Label and date each tube. Take one dehydrated bacteria disk from its stock bottle and drop into the corresponding tube. This is most easily done by heating an inoculating needle in a flame and, while hot, touching one of the disks. The disk will stick to the hot needle and can be

brushed off into the culture tube. To avoid contamination, sterilize the needle between different bacteria. If using the Bactrol Plus cultures, take 0.25ml sterile TSB from culture tube, mix gently in vial and add contents back into tube.

Every week to ten days, prepare a new batch of Tryptic Soy Broth as directed above. Label and date the tubes. Transfer the cultures from the old tubes into the new ones by dipping an inoculating loop into the old culture and swirling it in the new one. Discard the loop after each tube has been transferred. Autoclave and discard the old cultures and tubes.

After several transfers (once a month) discard the stock cultures and start fresh ones as directed above.

Preparation of Media

As discussed above, media must be prepared in glassware that is thoroughly washed and rinsed. This is to avoid any possible contamination of the media with chemicals that might promote or retard bacterial growth. Similarly, use only **reagent** grade water.

Determine the amount of media you will be preparing. Remember to add 50ml to the total - 20ml for the pH check and 30ml for the three quality control tubes. Choose a beaker of sufficient volume so it will not be more than **half** full.

Determine the weight of dehydrated media needed by multiplying the volume desired (in **liters**) times the weight of media required to prepare one liter. For example, you want 600ml of EC media. It takes 37.00g of dehydrated media to make one liter of EC broth. Since you want only 600ml of prepared media, multiply 37.00g by 0.6 (600ml = 0.6liter). You need to measure out 22.20g (37.00 x 0.6 = 22.20) of dehydrated media.

Weigh out the correct amount of media, using the Ohaus scale and a weighing boat. Put the dehydrated media into the appropriate beaker, add a stirring stone, and the correct volume of water. If opening a new container of media, be sure to date it. Invert the media container when placing it back on the shelf.

Place the beaker on a hot plate. Turn on the stirring bar so that it is rotating nicely. Turn on the heat to a fairly low setting to speed up the dissolving of the media. Check to be sure that no undissolved media is sticking to the sides of the beaker.
While media is dissolving, set up tubes in racks. Add inverted vials if necessary. Pipette media into culture tubes, cap and autoclave as directed below.

After cooling, check for the presence of bubbles in vials for those tubes that have them. Discard any tubes with bubbles. Check to be sure that caps on screw-top tubes are tight. Culture tubes with loose-fitting caps can be stored in a cool, dark cupboard for up to two weeks. Screw-cap tubes can be stored under similar conditions for up to three months. Label and date all media prior to storage.

The following is a list of the most commonly used media and the amounts for each. Remember:

- USE ONLY REAGENT (DI) WATER
- RECORD THE DATE WHEN BOTTLE IS OPENED
- INVERT MEDIA BOTTLE AFTER REMOVING NEEDED MEDIA

<u>Lauryl Tryptose Broth (LTB)</u> <u>Standard Plate Count Agar (SPCA)</u>

1x 35.60g/1000ml
17.80g/500ml
8.90g/250ml

2x 71.20g/1000ml
35.60 g/500ml
17.80 g/250ml

Sterilize 15 min. at 121°C, 15 psi

Brilliant Green Bile (BGB)

1x 40.00g/1000ml
20.00g/500ml
10.00g/250ml

Sterilize 15 min. at 121°C, 15 psi

EC

1x 37.00g/1000ml
18.50g/500ml
9.25g/250ml

Sterilize 15 min. at 121°C, 15 psi

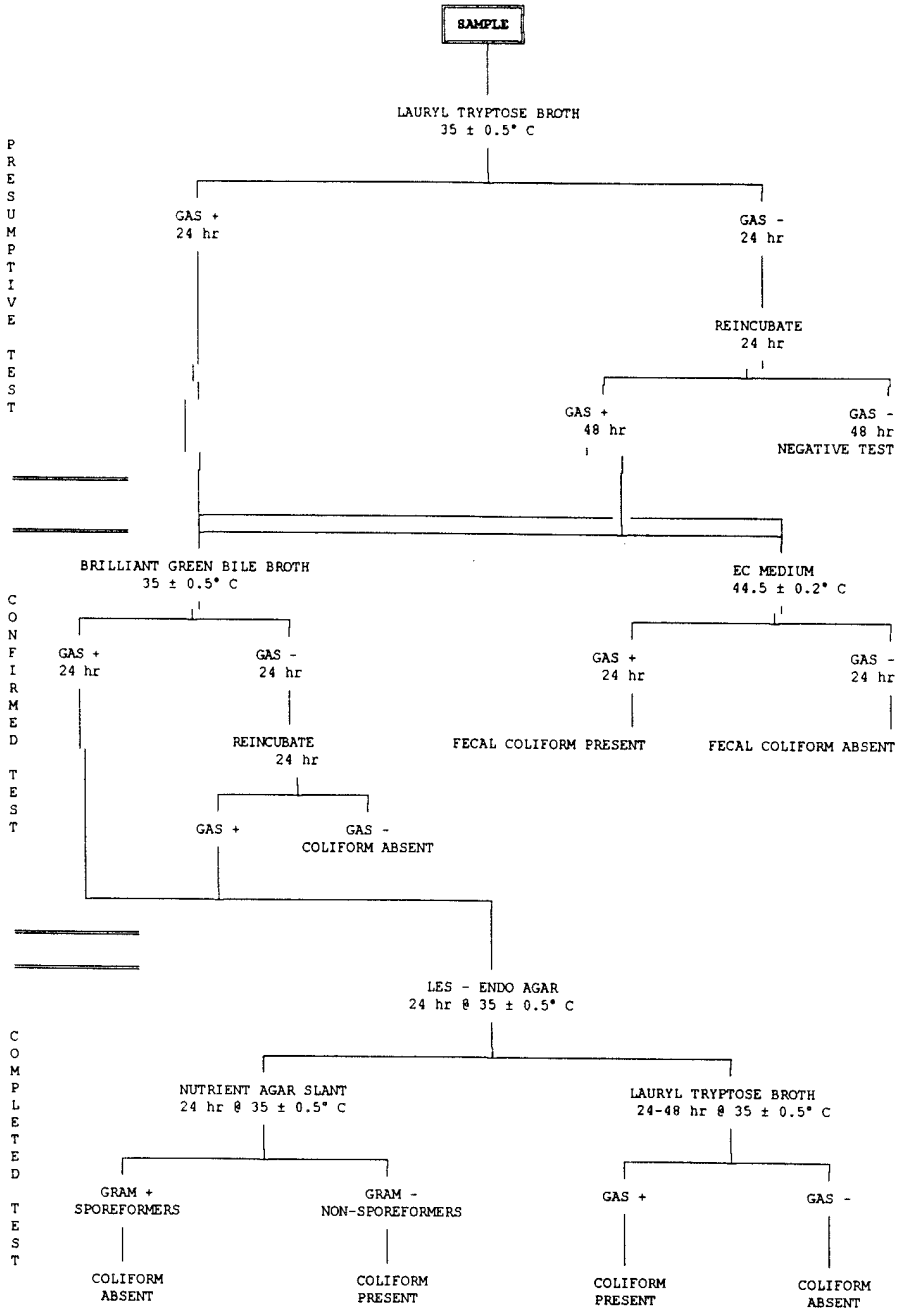
1x 23.50g/1000ml
11.75g/500ml
5.87g/250ml

Sterilize 15 min. at 121°C, 15 psi

Tryptic Soy Broth (TSB)

1x 30.00g/1000ml
15.00g/500ml
7.50g/250ml

Sterilize 15 min. at 121°C, 15 psi


SAMPLE
LAURYL TRYPTOSE BROTH
35 ± 0.5° C
PRESUMPTIVE TEST
GAS +
24 hr
GAS -
24 hr
REINCUBATE
24 hr
GAS +
48 hr
GAS -
48 hr
NEGATIVE TEST
BRILLIANT GREEN BILE BROTH
35 ± 0.5° C
EC MEDIUM
44.5 ± 0.2° C
CONFIRMED TEST
GAS +
24 hr
GAS -
24 hr
GAS +
24 hr
GAS -
24 hr
REINCUBATE
24 hr
FECAL COLIFORM PRESENT
FECAL COLIFORM ABSENT
GAS +
GAS -
COLIFORM ABSENT
LES - ENDO AGAR
24 hr @ 35 ± 0.5° C
COMPLETED TEST
NUTRIENT AGAR SLANT
24 hr @ 35 ± 0.5° C
LAURYL TRYPTOSE BROTH
24-48 hr @ 35 ± 0.5° C
GRAM +
SPOREFORMERS
GRAM -
NON-SPOREFORMERS
GAS +
GAS -
COLIFORM
ABSENT
COLIFORM
PRESENT
COLIFORM
PRESENT
COLIFORM
ABSENT

Multiple Tube Fermentation Process

Test for Coliform Bacteria

Much of the information in this section of the manual is taken from Standard Methods..., Edition 18, Section 9221 (p. 9-45) and Microbiological Methods for Monitoring the Environment, Water and Waste, p. 114. It is strongly recommended that the beginning laboratory technician study these sources in addition to the following discussion. Even experienced technicians may want to review this material from time to time.

General Discussion

The test for coliform bacteria is used to measure the suitability of a water for human use. The bacteria detected by this test are normally found in the intestinal tract of humans and other mammals. These bacteria are therefore present in sewage, numbering as many as 1,000,000 per milliliter. Coliform bacteria are considered to be harmless. However, their presence does indicate the possible presence of other pathogenic organisms. As a consequence, when coliform bacteria are found, the water is suspected of being polluted by human waste discharge.

Depending on the use of the water, standards are established for the numbers of coliform bacteria permissible in a given volume of that water. For example, a safe bathing water standard would not be as strict as a safe drinking water standard. The test is not only useful in determining the bacterial quality of a finished water, but also can be used by the operator in the treatment plant as a guide in achieving a desired degree of treatment.

The test for coliforms is a three-step process consisting of the presumptive phase, the confirmed phase, and the completed phase. The entire process is outlined in Figure 2.

Multiple-tube Fermentation Technique

Coliform bacteria are detected by placing an aliquot of the sample in Lauryl Tryptose Broth (LTB). The coliform bacteria are those which will grow in this medium producing gas by fermenting available sugars at 35°C within 48 hours. Thus, to detect these bacteria, the operator need only inspect fermentation tubes for gas. Coliform bacteria also change the pH of the media. An alternative method for detecting the growth of coliform bacteria involves adding bromcresol purple to the LTB media. A change in color to yellow indicates increasing acidity and the possible presence of lactose fermenting bacteria. In this lab, the use of fermentation tubes is the preferred method.

In practice, several fermentation tubes are used for each sample. Treated water samples may be tested by adding 10ml samples to ten tubes of double strength LTB. Effluent samples are subjected to a 15 tube dilution series (five tubes at each of three decimal dilutions). The most frequently used series involves 10ml, 1.0ml and 0.1ml aliquots of sample.

Recording of Most Probable Number

An estimation of the coliform bacterial density can be determined from the number of positive tubes in each series. This estimation is referred to as the **M**ost **P**robable **N**umber (MPN) and is reported as the number of coliform group organisms per 100ml of sample. It must always be kept in mind that this is strictly a statistical figure and not a precise enumeration.

Tables of MPN's are kept accessible in the laboratory and as Figure 3 in this manual. Additional tables with 95% confidence levels can be found on pages 9-49 and 9-50 of Standard Methods..., 18th Edition. A glance at the 95% confidence level will reveal how imprecise the MPN is.

To determine the MPN, record the number of positive tubes, then look up the number on the chart. For the ten-tube test of treated water samples, this is straightforward. For the fifteen tube dilution series it becomes a bit more complicated. Record the positive tubes for each five-tube dilution separately and report them starting from the largest sample to the smallest. Usually, the dilution series will consist of aliquots of 10ml, 1.0ml and 0.1ml. If all five of the 10ml tubes turn positive (presence of gas), three of the 1.0ml tubes, and one of the 0.1ml tubes are positive, this is reported as 5-3-1. This gives a MPN value of 110 from the chart.

The quantities indicated at the heads of the columns are for the common 10.0-1.0-0.1ml series of aliquots. The same values may be used in computing the MPN in larger or smaller portion plantings in the following manner: if, instead of 10.0-1.0-0.1ml portions, a combination of 100.0-10.0-1.0ml is used, the MPN is recorded as 0.1 times the value given in the tables. If, on the other hand, portions of 1.0-0.1-0.01ml are planted, the value recorded would be 10 times that given in the tables.

The general formula for determining the MPN value is:

MPN/100 ml = MPN value (from table) x (10/largest volume tested)

When more than three dilutions are employed in a decimal series, the results from only three consecutive dilutions are used in determining the MPN. To select which three dilutions to use, the highest dilution (smallest sample volume) which gives positive results in all five tubes tested (no lower dilution giving any negative results) and the next succeeding higher dilutions should be chosen. The results at these three dilutions should then be used in computing the MPN. For a complete discussion on determining MPN when more that three dilutions were tested, see Standard Methods..., 18th Edition, page 9-49 and following.

<u>Presumptive Phase</u>

The presumptive phase uses ten tubes of double strength LTB for each treated water sample or a fifteen tube dilution series for each effluent sample. Prepare the double strength LTB [LTB (2X)] as directed above and pipette 10ml portions into 18x150mm culture tubes with inverted ½ dram vials in the bottom. Cap tubes. Prepare single strength LTB [LTB (1X)] as directed above and pipette 10ml portions into 16x125mm tubes with inverted ¼ dram vials in the bottom. Cap tubes. Autoclave all tubes at 121°C for 15 minutes. After cooling, check the vials for bubbles (does not happen often). Discard if bubbles present.

For each treated water sample set up a row of ten LTB (2X) tubes. Label the first tube of each row with the lab number (and location, if desired) of the sample to be tested. For each raw, filtered or effluent sample set up a row of five LTB (2X) tubes and two rows of five LTB (1X) tubes. Label the first tube of each row with the lab number of the sample. Also indicate the amount of sample being tested on the 1X tubes.

Shake each sample bottle approximately 25 times. Add 10ml of sample to each of the treated tubes (a total of 100ml of sample). For the dilution series add 10ml of sample to each of the 2X tubes. Switch to a 1.0ml pipette. Add 1.0ml of sample to each of the first row of 1X tubes and 0.1ml of sample to each of the second row of 1X media (a total of 55.5ml of sample).

Place the inoculated tubes into a 35°C incubator. After 24 ± 2 hours, check all sample tubes for the presence of gas. Record results on the lab slip. Treat all positive tubes to the confirmatory phase discussed below. Return all other tubes to the incubator. After 48 ± 3 hours (from start

of test), check again. Record results and transfer all positive tubes. This completes the presumptive phase of the test.
Autoclave all LTB tubes for 30 minutes at 121°C to destroy any bacteria growing in the cultures. Discard medium and vial in each tube. Wash tubes in the dishwasher.

Notification of Positive Test
If any **treated** (drinking water) samples are positive during the presumptive phase, notify the appropriate people in the Park, at Region and at the Public Health Service. A list of people and phone numbers is found in Section 1 of this manual.

Repeat Sample vs. Resample
If any treated samples are positive, **three** repeat samples must be obtained immediately. These samples should come from the positive sample point, plus one upstream and one downstream sample, if possible. Repeat sample points are specified in the Water Systems Manual. Additionally, there must be **five** samples from this water system during the month following a positive test. This will mean additional sampling for the smaller systems.

If any treated samples exhibit turbid (excessively cloudy) tubes during the presumptive test, a resample from the same sampling point must be obtained immediately. Resampling will continue until the turbidity problem is resolved.

Notification and resampling procedures are given in the flow chart found in Figure 1 of Section 1.

Confirmed Phase
Cultures from each positive tube from the presumptive phase will be transferred to tubes of BGB and EC media. Prepare media as directed above and pipette 10ml portions into screw-top culture tubes with inverted ¼ dram vials. Autoclave at 121°C for 15 minutes. After cooling, check for bubbles in vials.

A culture from each positive LTB tube is transferred to both a BGB and an EC tube. Label each tube with the lab number, original sample amount (not necessary for treated samples), and number of tube in series. Dip a sterile swab or inoculating loop at least 3-5mm into the LTB tube and swirl it in an EC tube. Repeat to inoculate a BGB tube. Always transfer to the EC tube first, then the BGB. Autoclave and discard medium and vials in all LTB tubes.

When all transfers are done, incubate the BGB tubes at 35°C. Check after 24 ± 2 hours and 48 ± 3 hours for the presence of gas. EC tubes go into the water bath at 44.5°C. Make sure the water level is deep enough to at least cover the media in the tubes. Incubate for 24 ± 2 hours only and check for the presence of gas. Record results on the lab slip. The MPN for the BGB tubes is recorded as total coliforms, while the MPN for the EC tubes is for fecal coliforms.

At the end of this phase, autoclave and discard all EC and BGB tubes. Screw-top culture tubes are not reusable.

Completed Phase
Double confirmation into BGB media for total coliforms and into EC broth for fecal coliforms (see Section 9221E of Standard Methods, 18th Edition, pp.9-52 to 9-53) is used. Consider positive EC broth elevated temperature (44.5C) results as a positive completed test response for fecal coliforms. (See Section 9221 B.3 on page 9-47 of Standard Methods, 18th Edition)

MPN Calculation Chart

0-0-0	<2	3-0-0	8	5-0-0	23	5-3-3	170
0-0-1	2	3-0-1	11	5-0-1	30	5-4-0	130
0-1-0	2	3-1-0	11	5-0-2	40	5-4-1	170
0-2-0	4	3-1-1	14	5-1-0	30	5-4-2	220
		3-2-0	14	5-1-1	50	5-4-3	280
1-0-0	2	3-2-1	17	5-1-2	60	5-4-4	350
1-0-1	4			5-2-0	50	5-5-0	240
1-1-0	4	4-0-0	13	5-2-1	70	5-5-1	300
1-1-1	6	4-0-1	17	5-2-2	90	5-5-2	500
1-2-0	6	4-1-0	17	5-3-0	80	5-5-3	900
		4-1-1	21	5-3-1	110	5-5-4	1600
2-0-0	4	4-1-2	26	5-3-2	140	5-5-5	≥1600
2-0-1	7	4-2-0	22	0	<1.1	5	6.9
2-1-0	7	4-2-1	26	1	1.1	6	9.2
2-1-1	9	4-3-0	27	2	2.2	7	12.0
2-2-0	9	4-3-1	33	3	3.6	8	16.1
2-3-0	12	4-4-0	34	4	5.1	9	23.0
						10	≥23.0

#____AUTOCLAVE RUN SHEET

DATE	ITEMS STERILIZED	STERIL TIME	TIME		ELAP TIME	MAX TEMP	MAX PRES	QC CHECKS				TECH
			IN	OUT				TAPE	CLOCK	SPORE +/-	SPORE REF #	

INVENTORY LOG

REF #	DATE REC	ITEM	QUANT	LOT #	MAN. EXP DATE	OPEN DATE	OPEN EXP DATE	DISPOSAL	
								DATE	AMOUNT

MEDIA LOG

DATE	MEDIA	REF #	GRAMS	MLS	#TUBES	pH	CONTROLS			TECH
							+	-	0	

pH METER

DATE	TECH	TEMP	SET PH 4 STD	SLOPE pH 10 STD	CALIB pH 7 STD	REF # REMARKS

SEMIANNUAL TEMPERATURE RECORD
INCUBATOR_____ WATERBATH_____ REFRIGERATOR_____

CERTIFIED THERMOMETER_____ INCUBATOR THERMOMETER_____
CORRECTION FACTOR_____ DATE_____
CERTIFIED THERMOMETER #_____

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